EXHIBIT A

Neil.Bartfeld

From: Beck, Brian [BBeck@atcc.org]
Sent: Tuesday, February 10, 2009 2:17 PM
To: Neil.Bartfeld
Subject: ATCC 25104

Dear Neil

Thermus aquaticus ATCC 25104 was deposited in the ATCC Bacteriology collection on December 30, 1968.

Regards,

Brian J. Beck, PhD
Bacteriology Program Manager
American Type Culture Collection
10801 University Blvd
Manassas, VA 20110

Phone: 703-365-2700 x2676
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BELGIAN CO-ORDINATED COLLECTIONS OF MICRO-ORGANISMS

BCCM/LMG bacteria catalogue - Search results

Last data update: 16 January 2009

Search on: LMG accession number *contains* 7561

4 strain(s) found - displaying strain(s) 1 through 4

Modify search criteria New search

Strain Number: LMG 7561.1 equivalent to strain(s) LMG 7561.2 LMG 7561

Speciesname: Bacillus licheniformis (Weigmann 1898) Chester 1901 AL

Strain Number: LMG 7561.2 equivalent to strain(s) LMG 7561.1 LMG 7561

Speciesname: Bacillus licheniformis (Weigmann 1898) Chester 1901 AL

Strain Number:	**LMG 7561** Add to cart
Speciesname:	Bacillus licheniformis (Weigmann 1898) Chester 1901 AL
Other collections' numbers:	Claus III;DSM 392
Restrictions:	Biohazard group 1
Biological origin:	field soil
Geographic origin:	Rothamsted United Kingdom
Depositor:	DSM
History:	<- 1986, DSM <- D.Claus
Conditions for growth on solid media:	Medium 1, 37°C

Strain Number:	**LMG 17561** Add to cart

Speciesname:	Campylobacter lari Benjamin, Leaper, Owen and Skirrow 1984 VL
Other collections' numbers:	Endtz 2323BVA;Vandamme R-315
Restrictions:	Not yet assigned
Biological origin:	shellfish
Geographic origin:	Netherlands
Depositor:	P.Vandamme University Ghent Belgium
History:	<- 1996, P.Vandamme University Ghent Belgium <- 1994, H.Endtz Erasmus Univ. and Univ. Hosp. Rotterdam The Netherlands. (1993-1994)
Conditions for growth on solid media:	Medium 49, 37°C, microaerobic (e.g. 6% O2 + 7,5% H2 + 4% CO2 + 82,5% N2)

Modify search criteria New search

EXHIBIT C

System. Appl. Microbiol. 24, 277–284 (2001)

http://www.urbanfischer.de/journals/sam

SYSTEMATIC AND APPLIED MICROBIOLOGY

Random Amplified Ribosomal DNA Restriction Analysis for Rapid Identification of Thermophilic Actinomycete-like Bacteria Involved in Hypersensitivity Pneumonitis

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Received May 3, 2001

Summary

Hypersensitivity pneumonitis (HP) is a pulmonary disease characterised by inflammation that can be caused by, amongst other substances, a subset of 4 thermophilic mycelial bacteria: *Saccharopolyspora rectivirgula*, *Saccharomonospora viridis*, *Thermoactinomyces sacchari*, and *Thermoactinomyces vulgaris*. Air sampling analyses in highly contaminated environments are often performed to evaluate exposure to these species which are difficult and fastidious to identify by conventional techniques. The aim of this study was to use amplified ribosomal DNA restriction analysis (ARDRA) to develop a method of identification for those thermophilic organisms that would be more rapid and simple. Strains of these 4 species were obtained from the American type culture collection (ATCC) and were characterized using biochemical tests and ARDRA patterns obtained on their partial-lenght amplified 16S rDNAs. To validate this approach, ARDRA with two restriction enzymes, *Taq*I and *Hha*I, was applied to 49 thermophilic actinomycete-like strains from environmental samples (sawmills). The results obtained show that combining some cultural characteristics and biochemical tests, such as xanthine or hypoxanthine decomposition, growth in the presence of NaCl, lysozyme or novobiocin, and spore resistance over 100 °C provide a rough identification and selection of the genera of interest. Consequently, target species could be confirmed by digestion of partial-lenght 16S rDNA with the use of *Taq*I and *Hha*I restriction enzymes that gave specific restriction patterns. ARDRA analyses on the 49 environmental actinomycete-like organisms revealed the presence of 8 *Saccharopolyspora rectivirgula*, 2 *Saccharomonospora viridis*, and 15 *Thermoactinomyces vulgaris* strains, the other strains had restriction patterns different than those of the species of interest. Results of the present study will be applicable to other potential HP environments such as dairy barns, peat bogs and compost plants.

Key words: ARDRA – thermophilic actinomycetes – hypersensitivity pneumonitis – environmental samples

Introduction

Some members of the genera *Saccharopolyspora*, *Saccharomonospora* and *Thermoactinomyces* are often responsible for hypersensitivity pneumonitis (HP) (MCNEILL and BROWN 1994), a respiratory disease caused by an allergic response to a variety of antigens such as bacteria, molds and animal proteins. Farmer's lung was the first type of HP described (CORMIER and LAVIOLETTE, 1996) and is still one of the most prevalent forms of the disease (LACEY 1971). In eastern Canada, farmer's lung is caused mostly by *Saccharopolyspora rectivirgula* (*Faenia rectivirgula*), a thermophilic actinomycete found in mouldy hay (DUCHAINE et al., 1999). The strains of three other thermophilic species, *Saccharomonospora viridis*, *Thermoactinomyces sacchari* and *Thermoactinomyces vulgaris*, may act as causative agents for this disease (CORMIER and LAVIOLETTE 1996; LACEY 1971).

Organisms from the genera *Saccharopolyspora* and *Saccharomonospora* are true actinomycetes (STACKEBRANDT et al., 1997) whereas *Thermoactinomyces* species produce endospores as shown in bacilli (CROSS et al., 1968). Nevertheless, *Thermoactinomyces* species morphologically resemble actinomycetes and form aerial and

0723-2020/01/24/02-277 $ 15.00/0

substrate mycelia. Based on 16S rRNA analysis, phylogenetic position of these bacteria is quite different: *Saccharopolyspora* and *Saccharomonospora* are the members of the order *Actinomycetales* (STACKERBRANDT et al., 1997) whereas *Thermoactinomyces* is more closely related to *Bacillus* species and should be placed within the family *Bacillaceae* (YOON and PARK 2000).

The identification of these microorganisms by traditional techniques is difficult and fastidious methods are needed to confirm the nature of a species. Furthermore, certain characteristics, such as aerial mycelium production, often vary with culture conditions. The filamentous growth and the growth temperature requirement of the causative bacteria of HP make their identification by commercial kits difficult. Several PCR-based DNA fingerprinting methods have been described to enable identification of cultured bacterial organisms to the species level in a relatively short period of time, this method offers obvious advantages over the more classic identification methods (VANEECHOUTTE et al., 1998; JENSEN et al., 1993; Widjojoadmotjo et al., 1994; JAYARAO et al., 1991). ARDRA (amplified rDNA restriction analysis) has been studied most thoroughly and has been used for the identification of species of different taxa (MATAR et al., 1993; VANEECHOUTTE et al., 1992; VANEECHOUTTE et al., 1995; VILA et al., 1996).

The aims of this study were to establish the amplified ribosomal DNA restriction analysis (ARDRA) patterns of four thermophilic bacterial species responsible for HP and to use these patterns to identify environmental strains isolated from sawmills (DUCHAINE et al., 2000). The overall approach of this study was to:

1. verify the response to biochemical tests of the strains of *Saccharopolyspora rectivirgula*, *Saccharomonospora viridis*, *Thermoactinomyces vulgaris* and *Thermoactinomyces sacchari* and determine their ARDRA patterns;

2. perform the first selected identification steps on environmental strains using selected biochemical tests and to complete the identification with ARDRA in order to quantify (in air samples) environmental HP causing thermophilic actinomycete-like bacteria.

Materials and Methods

Microorganisms and growth conditions

Three or four ATCC strains (including type strains) of 4 target species responsible for HP were used in these experiments: *Saccharopolyspora rectivirgula* (ATCC 33515T, 15347 and 29034), *Saccharomonospora viridis* (ATCC 15386T, 15735 and 15736), *Thermoactinomyces sacchari* (ATCC 27375T, 27349 and 27376) and *T. vulgaris* (ATCC 43649T, 15733, 15734 and 21364). Forty nine environmental strains isolated from air samples of 17 sawmills in Eastern Canada were used as environmental strains to be identified (DUCHAINE et al., 2000). All the strains were cultured at 52 °C for 7 to 15 days on Trypticase soy agar (TSA) (Difco Laboratories, Detroit, Mich.), Nutrient agar (NA) (Difco). NA supplemented with 1% glucose (NA+1%G) was used for *T. sacchari* strains.

Physiological and growth properties of ATCC strains

Experimental procedures for physiological growth properties analysis were performed as previously published (DUCHAINE et al., 1999).

- **Optimal growth temperatures:** For each ATCC strain, TSA, NA and NA+1% glucose were inoculated on slant media with fresh cultures and incubated at 25, 37, 45, 52 and 60 °C. Growth intensity was scored after 7 days (0 = no growth to 3 = good growth).

- **Growth in the presence of NaCl:** TSA or NA+1%G supplemented with 7 or 10% NaCl were inoculated with fresh culture in a central trait. After 7, 14 and 21 days, growth was observed (0 = no growth to 3 = optimal growth) and the presence (+) or absence (–) of aerial mycelium was noted.

- **Casein decomposition:** This test was performed according to GORDON et al., (1974). Plates were inoculated and incubated for 28 days. The presence (+) or absence (–) of a zone of hydrolysis surrounding the growth was noted.

- **Tributyrin hydrolysis:** Isolated colonies were obtained on Tributyrin agar (Oxoid, Basingstoke, Hants, England). After 28 days of incubation, the presence (+) or absence (–) of a zone of hydrolysis surrounding the growth was noted.

- **Tyrosine, xanthine and hypoxanthine hydrolysis:** These tests were performed according to GORDON et al., (1974) except that TSA and NA+1%G were used instead of the basal medium (KURUP and FINK 1975). Fresh cultures were inoculated twice (once on the surface of the medium and once into the medium) and incubated for 28 days. The presence (+) or absence (–) of a zone of hydrolysis surrounding the growth was noted.

- **Esculin and arbutin hydrolysis:** For these tests, 1 g of esculin or arbutin and 0.5 g of ferric citrate were added to 1 liter of TSA or NA + 1%G before sterilization (KURUP and FINK 1975). The plates were inoculated and a black-brown zone around the colonies indicated degradation (+).

- **Starch hydrolysis:** This test was performed according to GORDON and MIHM (1957) except that, here again, TSA and NA+1%G were instead of the basal medium (KURUP 1975). Plates were inoculated with a swab and incubated for 7, 14, 21 and 28 days before plates were flooded with Gram's iodine solution(KURUP 1975). A clear zone (+) surrounding the growth indicated starch hydrolysis.

- **Presence of DNAses:** DNAse Test Agar (Difco) was used to demonstrate DNAse activity. Plates were inoculated and incubated for 7 and 14 days before dishes are flooded with 1 N HCl. If DNA was hydrolysed, a clear zone appeared (+) around the growth.

Resistance to antibiotic, lysozyme and heat

Experimental procedures for antibiotic, lysozyme and heat resistance analysis were performed as previously published (DUCHAINA et al., 1999).

- **Novobiocin:** This test was performed according to KURUP and FINK (1975). Briefly, a 10 mg/ml novobiocin solution was sterilized by filtration (0,22 µm) and added to Tryptic Soy Broth (TSB) (Difco) or TSB+1%G for a final concentration of 30 µg/ml. Five ml of TSB ± 1% G+novobiocin and TSB ± 1% G were inoculated with 100 µl of fresh culture and growth was evaluated (+ or –) after 7 and 14 days.

• **Lysozyme:** This test was performed according KURUP and FINK (1975) and GORDON (1966). Five ml of a 1mg/ml of filter-sterilized (0,22 µ) lysozyme solution in 0.01N HCl was added to 100 ml of TSB + 1%G + glycerol (7%). Five milliliters broth aliquots were inoculated with 100 µl of a TSB + 1% G+glycerol (7%) culture. Growth in the presence of lysozyme was observed or not (+ or –) after 14, 21 and 28 days.

• **Heat resistance:** TSA or NA + 1% G plates were inoculated with liquid cultures (TSB + 1% G) that had been boiled for 10, 30, 60 or 120 min. Plates were incubated for 7 and 14 days and growth was observed or not (+ or –).

Genetic analysis

• **DNA isolation:** Since some gram-positive bacteria are very difficult to lyse, genomic DNA was extracted using a combination of 2 protocols (YOON et al., 1996; LORTIE et al., 1994). Frozen cultured cells were washed in 0.01 M Tris-HCl, pH 8.0 before resuspension in 0.02 M Tris-HCl, pH 8.0 with 50% polyethylene glycol (PEG) (3400 molecular mass) diluted in H_2O (24% final). A 1 minute ultrasonic treatment was performed on the cells prior to incubation of 1 hour at 37 °C. The cells were then centrifuged (9 000 rpm) and resuspended in 0.1 M Tris-HCl, pH 8.0, 1% SDS (sodium dodecyl sulfate), 0.1 M EDTA before a 15 minute incubation at 37 °C. Cells underwent 5 freeze-thaw cycles in dry ice/ETOH/warm water of 1 minute followed by phenol-chloroform (Sigma, St-Louis, MO) extraction. DNA was precipitated in 1 volume of isopropanol and resuspended in a minimal volume of 0.01 M Tris-HCl, pH 8.0. RNA was removed by digestion with 20 µg/ml of RNase A (Boehringer Mannheim, Mannheim, Germany) for 1 hour at 52 °C and protein was digested with 100 µg/ml of proteinase K (Boehringer Mannheim) for 1 hour at 37 °C. The DNA was then reprecipitated in 2 volume of isopropanol, dissolved in a minimal volume of 0.01 M Tris-HCl, pH 8.0 and frozen at –20 °C.

• **PCR amplification:** The PCR was performed according to MCVEIGH et al., (1996) except that with the annealing temperature of 68 °C. The primers used specifically amplified partial-length 16S rRNA gene from actinomycetes and related genera. Although corresponding regions of published 16S rDNA sequences of *Thermoactinomyces* strains differed by one or two bases from the one of the primers, these primers were successfully applied without modifications to all *Thermoactinomyces* strains under study. The primers are as follows: ACT283F (5'-GGGTAGCCGGCCUGAGAGGG) corresponding to *E. coli* 16S rRNA positions 283 to 302 and ACT1360R (5'-CTGATCTGCGATTACTAGCGACTCC) corresponding to the complement of *Escherichia coli* 16S rRNA positions 1360 to 1336. For the ATCC strains, 10 PCR reactions were pooled whereas 3 PCR reactions were pooled for the environmental strains. If the gel showed more than one PCR product, the target band (about 1050 base pairs (bp)) was purified using the QIAquick gel extraction kit (Qiagen).

ARDRA

Enzymes with 4-6 base long recognition sites were used: *Ava*I, *Bsm*FI, *Dpn*I, *Dde*I, *Eco*RI, *Fok*I, *Hha*I, *Mse*I, *Sau*96I, *Taq*I (New England Biolabs (NEB), Mississauga, ON), *Alu*I, *Eco*RII, *Hinf*I, *Rsa*I, (Gibco BRL, Grand Island, NY), and *Ban*II (Boehringer Mannheim). The amount of DNA used was adjusted according to the concentration estimation related to the gel band intensity. Aliquots of 10–12 µl PCR products were digested with these restriction enzymes (1.5 U) at 37 °C for at least 1h. The restricted DNA was separated on 3% agarose gel containing ethidium bromide.

• **Cloning and sequencing of PCR products:** PCR products of 16S rRNA genes of *T. sacchari* ATCC 27349 and *T. vulgaris* ATCC 15734 were cloned according to the TOPO TA cloning kit protocol (Invitrogen Corporation, Carlsbad, CA) and the plasmid DNA was extracted according to QIAprep spin miniprep kit protocol (Qiagen). Both strands of cloned 16S rDNA fragment were sequenced by the automatic sequencing service of Laval University (Laval university, Ste-Foy, Quebec). Analysis of the sequences were performed with the Genetic Computer Group package (GCG (9.1)).

• **Phylogenetic analysis of 16S rDNA sequences:** Multiple alignment of sequences was carried out using the Clustal W package (THOMPSON et al., 1994). Distance analyses using JUKES & CANTOR (JUKES and CANTOR 1969) correction and bootstrap resampling (1000 data sets) were done using the TREECON package (VAN DE PEER and DE WACHTER, 1994) while phylogenetic tree was generated using the neighbour-joining method (SAITOU and NEI, 1987).

• **GeneBank accession numbers:** Genbank accession number for the 16S rDNA sequences of *Thermoactinomyces sacchari* ATCC 27349 and *Thermoactinomyces vulgaris* ATCC 15734 are AF089890 and AF089892, respectively.

Results

Biochemical studies

Growth was observed between 37 and 60 °C for all ATCC strains except for *S. viridis* ATCC 15736 (25 to 52 °C), *T. sacchari* ATCC 27349 (37 to 52 °C) and *T. sacchari* ATCC 27376 (45 to 60 °C). Since 52 °C was determined as the optimal growth temperature of the ATCC strains, environmental strains were isolated, grown and biochemically characterized at this temperature. Colony morphology and pigmentation facilitated the isolation of *S. rectivirgula*-like strains (orange-yellow to orange-brown colonies), of *T. sacchari* (rapid autolysis of its colonies) and of *T. vulgaris* (large spreading colonies with white aerial mycelium). Some strains of *S. viridis* produced a green-black pigment at 52 °C in the culture media and/or on its aerial mycelium. Those characteristics allowed us to isolate suspected organisms of the 4 species of interest. The isolation of environmental strains was very strict; every suspected colony or colony of unknown identity was isolated. Following the isolation of thermophillic strains, simple selected tests were performed. Data compiled in Table 1 show that *S. rectivirgula* could be recognized by its colony color, its growth in presence of 7% or 10% NaCl with production of white aerial mycelium (DUCHAINE et al., 1999). Spores survival at 100 °C was verified for strains exhibiting white aerial mycelium in order to differentiate *Thermoactinomyces* sp. from other genera. Resistance to lysozyme was performed on heat spore resistant strains to discriminate *T. vulgaris* preferentially from *T. sacchari*, the latter being sensitive. As shown in Table 1, some properties namely hydrolysis of tyrosine, esculin or starch as well as novobiocin or heat resistance, were variable within the species *T.sacchari* and *T. vulgaris*.

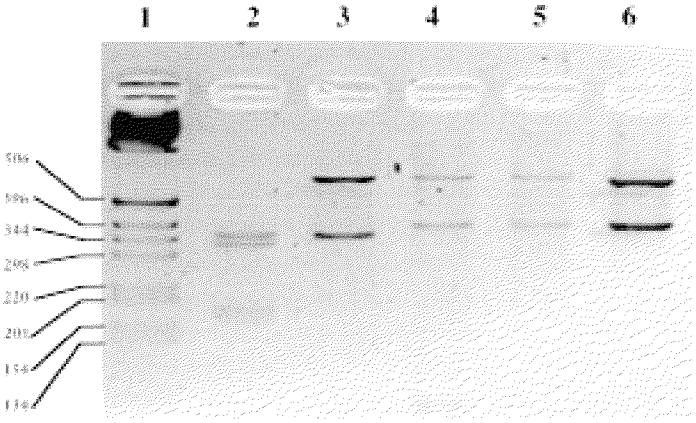

Fig. 1a. Amplified 16S rDNA from some strains of *Saccharopolyspora rectivirgula, Saccharomonospora viridis, Thermoactinomyces sacchari* and *Thermoactinomyces vulgaris* digest with *Taq1*. Lanes: 1, 1 Kbp ladder (134, 154, 201, 220, 298, 344, 396 and 506 bp); 2, *S. rectivirgula* ATCC 33515T, 15347 or 29034 (360, 330, 180 and 160 bp bands); 3, *S. viridis* ATCC 13586T, 15735 or 15736 (660 and 360 bp bands); 4, *T. sacchari* ATCC 27375, 27349 or 27376 (680 and 400 bp bands); 5, *T. vulgaris* ATCC 43649T (680 and 400 bp bands); 6, *T. vulgaris* ATCC 15733, 15734 or 21364 (650 and 400 bp bands).

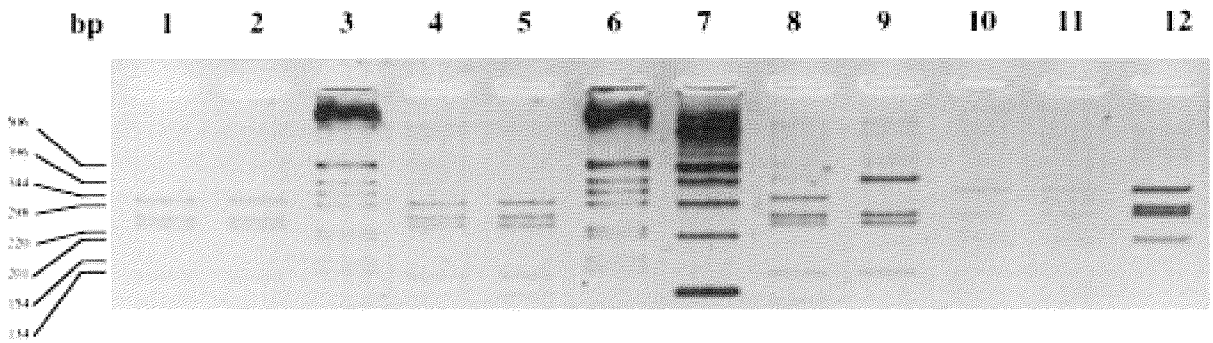

Fig. 1b. Amplified 16S rDNA from some strains of *Saccharopolyspora retcivirgula, Saccharomonospora viridis, Thermoactinomyces sacchari* and *Thermoactinomyces vulgaris* digested with *Hha*I. Lanes: 1, 2 and 8, *S. retivirgula* ATCC 15347, 29034 and 33515T, respectively (310, 250, 230, 120 and 70 bp bands); 4 and 5, *S. viridis* ATCC 17735 and 15736, respectively (300, 250, 230, 120 and 70 bp bands); 9, *S. viridis* 15386T (400, 250, 230 and 120 bp bands); 10, *T. sacchari* ATCC 27349 (340, 250, 180 and 100 bp bands); 11, *T. vulgaris* ATCC 43649T (340, 300, 250 and 180 bp bands); 12, *T. vulgaris* ATCC 15733 (340, 270, 250 and 180 bp bands); 3 and 6, 1 Kbp ladder (506, 396, 344, 298, 220, 201, 154, and 134 bp); 7, 100 bp ladder (500, 400, 300, 200, 100 bp).

ARDRA analysis

Among 15 restriction enzymes tested (see Materials and Methods), *Taq*I and *Hha*I were the most suitable for the differentiation of the 4 species under study. *Hha*I and *Taq*I restriction profiles obtained in this study for *S. viridis* and *T. vulgaris* type strains were in accordance with the restriction profiles expected from a computer-simulated digestion of the published 16S rDNA sequences of these strains. Three strains of *S. rectivirgula* namely ATCC 33515^T, ATCC 15347, ATCC 29034 showed similar profiles with *Hha*I as well as *Taq*I (Fig.1). *Hha*I produced two patterns for the three strains of *Saccharomonospora viridis* (ATCC 15386^T differed from ATCC 15735 and ATCC 15736) whereas *Taq*I patterns of all *Saccharomonospora viridis* strains were identical. *T. vulgaris* strains showed two patterns with *Hha*I as well as with *Taq*I and, in both cases, the type strain ATCC 43649^T differed from the other studied strains of this species. The restriction profiles obtained with *Taq*I and *Hha*I for *T. sacchari* ATCC 27349 and for type strain of *T. sacchari* (Fig.1) differed. ARDRA patterns obtained with *Taq*I and *Hha*I allowed the differentiation of type strains of all species under study (Figure 1a, 1b). The ARDRA method set with ATCC strains was used to identify 49 environmental strains isolated from sawmills. We identified 15 strains of *T. vulgaris*, 8 strains of *S. rectivirgula*, and 2 strains of *S. viridis* (Table 2). Restriction profiles with *Hha*I and *Taq*I of one environmental isolate, N-79$_{s15}$, was the same as for *T. sacchari* ATCC 27349 but no environmental isolate showed similar restriction profiles as the ones expected for type strain of *T. sacchari*. The other 23 environmental strains showed different ARDRA patterns than those of *S. rectivirgula*, *S. viridis*, *T. vulgaris* and *T. sacchari* strains examined in this study.

16S rRNA analysis

Partial-length 16S rDNA sequences of *T. vulgaris* ATCC 15734 (1052 bp) and *T. sacchari* ATCC 27349 (1077 bp) were determined. *T. vulgaris* ATCC 15734 was closely related to the type strain of *Thermoactinomyces sacchari* and *T. sacchari* ATCC 27349 was distantly clustered with type strains of *T. intermedius* and *T. vulgaris* (Fig. 2).

EXHIBIT C

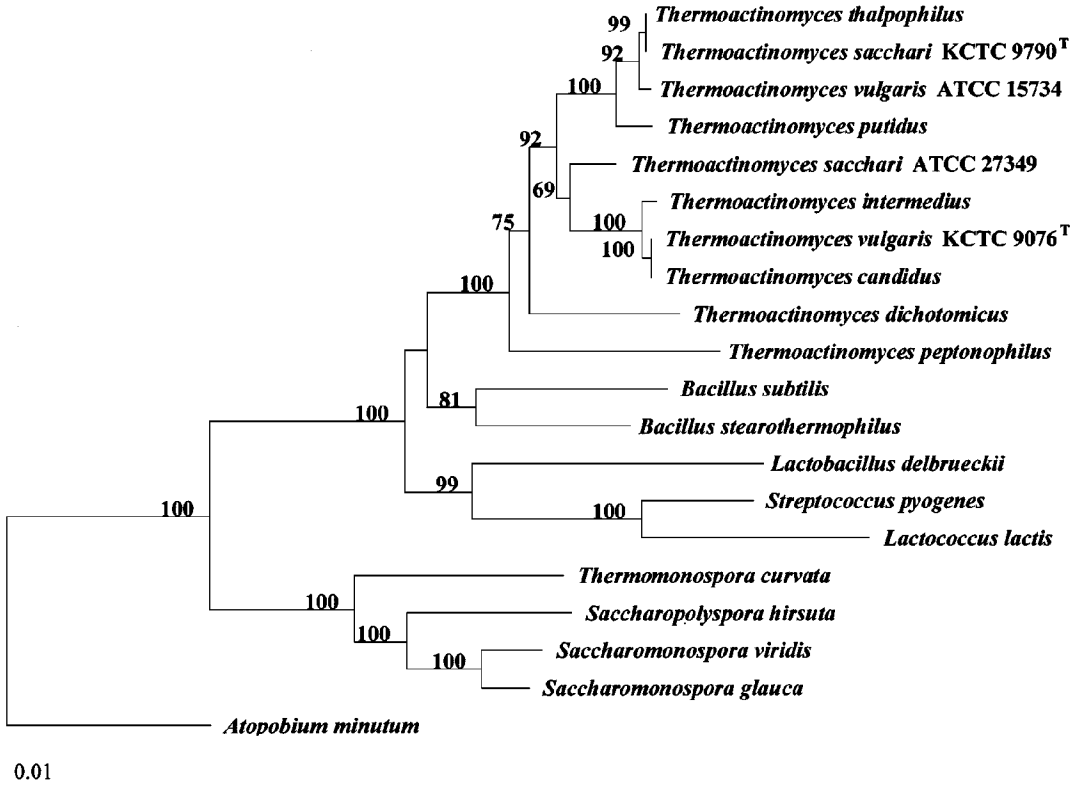

Fig. 2. Phylogenetic tree showing the positions of *Thermoactinomyces vulgaris* ATCC 15734 and *Thermoactinomyces sacchari* ATCC 27349 and representatives of some other taxa based on 16S rDNA sequences. The tree was built with the neighbor-joined method by using the Jukes-Cantor distance estimation. The scale bar represents 1 nucleotide substitution per 100 nucleotides. Bootstrap values greater than 50% are shown at the branch points.

Discussion

The aim of the present study was to develop a molecular-based strategy to rapidly and accurately identify thermophilic actinomycete-like bacteria that can induce HP and to use this method to identify environmental strains. A few biochemical tests was carried out to screen for HP-inducing strains prior to ARDRA analyses. Tests such as colony color and morphology, growth in the presence of NaCl, and spore heat resistance are examples of simple tests that allow the selection of strains of interest. The following ARDRA will confirm or infirm the taxomonic identification of the isolated strains to the species level.

The most difficult step in ARDRA is the DNA isolation. For some gram-positive organisms that show very resistant cell wall structures, the DNA isolation is fastidious and cell lysis requires various steps. Furthermore, the fastidious growth of some organisms such as *T. sacchari* made it difficult to harvest large cell volume to extract DNA. Nevertheless, the method chosen was successful to extract DNA from all strains used in this study.

The results of our study indicate that ARDRA is a fast method that can easily be used to clearly identify thermophilic actinomycete-like organisms that could induce HP in a given environment. Type strains of *Saccharopolyspora rectivirgula, Saccharomonospora viridis, Thermoactinomyces vulgaris* and *Thermoactinomyces sacchari* are easily differentiated from each other by *Taq*I and *Hha*I restriction profiles of amplified 16S rDNA fragments. However, ARDRA analyses with these restriction enzymes indicated some heterogeneity of the 16S rRNAs within *S. viridis* strains and showed that some of non-type strains of *T. sacchari* and *T. vulgaris* studied in our study did not seem to belong to the corresponding species. For example, *T. vulgaris* ATCC 15733 had *Taq*I and *Hha*I restriction patterns characteristic for type strain of *T. sacchari.* Biochemical tests also indicated heterogeneity among the strains of these two species (Table 2). Amplified 16S rDNA fragments of *T. vulgaris* ATCC 15734 (1052 bp) and *T. sacchari* ATCC 27349 (1077 bp) were sequenced in order to confirm the taxonomic position of these strains. *T. vulgaris* ATCC 15734 was shown to be closely related to *T. sacchari,* whereas *T. sacchari*

Table 1. Cultural and biochemical characteristics of the bacterial species involved in hypersensitivity pneumonitis.

Tests	Investigated species			
	Saccharopolyspora rectivirgula 3 strains	*Saccharomonospora viridi* 3 strains	*Thermoactinomyces sacchari* 3 strains	*Thermoactinomyces vulgaris* 4 strains
Growth with 7% NaCl	3/M[1]	3/0	0/0	0/0
Growth with 10% NaCl	3/M[1]	3/0	0/0	0/0
Casein decomposition	3	3	3	4
Tributyrin hydrolysis	3	3	3	4
Tyrosin hydrolysis	3	3	0	3[3]
Xanthin hydrolysis	3	0	0	0
Esculin hydrolysis	3	3[2]	3	1[3]
Hypoxanthin hydrolysis	3	0	0	0
Starch hydrolysis	0	3	3	3[3]
DNAses	3	3	3	4
Novobiocine resistance 30 µg/ml	0	0	2	4
Lysozyme resistance	0	0	0	4
Heat resistance (100 °C)				
10 minutes	0	0	2	4
30 minutes	0	0	2	4
60 minutes	0	0	2	4
120 minutes	0	0	1	3
Arbuting splitting	2	0	3	1[3]

[1]M – presence of aerial mycelium; 0 – absence of aerial mycelium
[2]strains were slightly positive
[3]only the type strain = 1[3]; except the type strain 3

Table 2. ARDRA patterns of environmental thermophilic actinomycete-like strains isolated from air samples of 17 sawmills in Eastern Canada.

Environmental strains*	Length in bp of ARDRA fragments with *Taq*I	Length in bp of ARDRA fragments with *Hha*I	Identifican of environmental isolates based on ARDRA patterns
N-27$_{s7}$, N-37$_{s3}$, N-49$_{s4}$, N-64$_{s10}$, T-8$_{s6}$, T-16$_{s7}$, T-20$_{s1}$, T-40$_{s10}$	160–180–330ä360	70–120–230–250–310	*Saccharopolyspora rectivirgula*
N-18$_{s6}$, N-21$_{s6}$, N-32$_{s1}$, N-38$_{s2}$, N-48$_{s4}$, N-55$_{s9}$, N-57$_{s9}$, N-59$_{s9}$, N-60s10, N-61$_{s10}$, N-72$_{s13}$, N-78$_{s15}$, N-83$_{s17}$, T-12$_{s6}$, T-32$_{s4}$	400–680	180–250–300–340	*Thermoactinomyces vulgaris*
N-36$_{s2}$, T-26$_{s1}$	360–660	120–230–250–400	*Saccharomonospora viridis*
N-79$_{s15}$	400–680	100–180–250–340	*Thermoactinomyces sacchari* ATCC 27349
N-7$_{s5}$, N-11$_{s5}$, N-34$_{s2}$, N-39$_{s2}$, N-40$_{s2}$, N-56$_{s9}$, T-3$_{s5}$, T-13$_{s6}$, T-19$_{s7}$, T-21$_{s1}$	360–660	230–250–330	Unknown
N-68$_{s11}$, N-69$_{s13}$	330–360–400ä660	90–160–250–420	Unknown
N-62$_{s10}$	210–320–360–400–680	160–190–260–340	Unknown
N-82$_{s17}$, T-63$_{16}$, T-65$_{s17}$	330–360	160–180–250–340	Unknown
T-28$_{s3}$	330–360	200–270–420	Unknown
N-15$_{s5}$, N-16$_{s5}$	330–360	160–180–250–330–400	Unknown
T-45$_{s11}$	330–260	230–250–?	Unknown
N-75$_{s14}$	330–400–660	180–250–300–340–420	Unknown
N-77$_{s15}$	180–400–500	180–250–300ä340	Unknown
N-9$_{s5}$	330–360	120–170–270–420	Unknown

* s(x) – sawmill number

EXHIBIT C

ATCC 27349 was distantly clustered with type strains of *T. intermedius* and *T. vulgaris* (Figure 2). *Thermoactinomyces sacchari* strain ATCC 27349 might represent a new species of the genus *Thermoactinomyces* since it shows a low level of 16S rDNA sequence similarity with all type strains from this genus. Our data, as well as recent 16S rDNA sequencing (YOON and PARK 2000) and DNA-DNA hybridization (YOON et al., 2000) studies, show the need of a detailed taxonomic investigation to improve the classification of the genus *Thermoactinomyces* as well as its species identification.

ARDRA patterns with *Taq*I and *Hha*I restriction enzymes for type strains of *S. rectivirgula, S. viridis, T. vulgaris* and *T. sacchari* were used in our study as references to identify environmental isolates. Among the 49 thermophillic actinomycete-like strains from environmental samples (sawmills), 26 strains were identified as possible members of the species *S. rectivirgula, S. viridis, T. vulgaris* and one isolate, N-79_{s15}, showed the same *Taq*I and *Hha*I restriction profiles as for *T. sacchari* ATCC 27349. Among the other 23 isolates, 10 remaind unidentified since their *Hha*I and *Taq*I restriction profiles differed from each other. Computer-simulated digestion of all available 16S rDNA sequences from the genera *Saccharopolyspora, Saccharomonospora* and *Thermoactinomyces* did not allow the identification of any of these strains.

Rather high percent, about 50%, of environmental isolates from air samples of 17 sawmills in Eastern Canada were identified as members of the four species responsible for hypersensitivity pneumonitis (HP). This indicates that a rough identification based on simple selected cultural and biochemical tests permits the selection of the genera of interest prior to ARDRA analysis of suspected isolates. Our findings will therefore be applicable to any environment that may have a potential or suspected risk for bacterial organisms that can induce HP.

Acknowledgements

This work was supported by the Institut de recherche en santé et sécurité du travail du Québec (IRSST) and by le Conseil national de recherche en sciences naturelles et en génie du Canada (CRSNGC/NSERC).

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EXHIBIT D

BP.PURA.017A-US.WO

4) METHOD FOR PRODUCING THERMITASE

7) The invention relates to the production of a thermophilic protease from Thermoactinomyces vulgaris. Thermitase is used for gluten breakdown in the production of processed foodstuff, waffles, and bakery products; the preparation of protein partial hydrolysates; and other processes. The method comprises inoculating an employed nutrient solution, which is saturated with oxygen, with spores for pre-cultivation and conducting the germination in a hermetically sealed vessel with a decrease of the oxygen partial pressure to 20 to 40 % of the oxygen partial pressure at saturation, maintaining the desired oxygen partial pressure, subsequently transferring the pre-culture into a fermenter with the production medium and carrying out the fermentation, additional quantities of nutrients being supplied in the process.

EXHIBIT D

BP.PURA.017A-US.WO

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METHOD FOR PRODUCING THERMITASE

SCOPE OF THE INVENTION

The invention relates to a method for producing a thermophilic protease from Thermoactinomyces vulgaris with special properties for the partial hydrolysis of animal and vegetable proteins. The enzyme thermitase being produced is suitable for use in various processes in the food industry as crude preparation or in partially purified form, and in highly purified form as fine biochemical. Fields of application are gluten breakdown in the production of processed foodstuff, waffles, and bakery products, the preparation of protein partial hydrolysates for certain target groups, for example for diet purposes or the nourishment of sick people and convalescent patients. The method for producing thermitase is suitable for the large-scale production of the enzyme in agitated fermenters.

CHARACTERISTICS OF THE KNOWN TECHNICAL SOLUTIONS

It is known that numerous microorganisms, for example those of the genus Aspergillus, Bacillus, and Streptomyces, under the respective required culture conditions, produce proteases, which differ from one another with respect to their properties. For different application purposes, it is advantageous if the activity of the enzyme is also or primarily effective at a higher temperature. It is further

known that by cultivating Thermoactinomyces vulgaris on suitable nutrient substrates, a thermophilic protease can be produced, the optimum temperature of which is 60 - 70 °C and which causes a largely non-specific and for this reason extensive cleavage of animal and vegetable proteins.

The known strain for producing thermitase from the species Thermoactinomyces vulgaris and a mutant thereof are deposited at the Central Strain Collection of the GDR in the Central Institute for Microbiology and Experimental Therapy in Jena under IMET 9512 and IMET 9515. The characteristics of the strain and the cultivation thereof for the purpose of the submerged preparation of thermitase are described in WP 112 662. In a patent of addition WP 117 083, the method has been improved in such a way that additions of rapeseed oil, dry yeast, and acetic acid to the nutrient medium result in an increase of the enzyme yield.

However, the reproducibility of the known methods is unsatisfactory since the germination of the microorganism spores used as inoculum in the pre-culture (shaking culture) used is strongly dependent upon the hydrodynamic conditions and differs with respect to the germination rate. As a result, growth retardations frequently occur in the production medium, which are accompanied by insufficient product formation and increased risk of infection.

It is known that the different behavior during spore germination is caused by the dependence of the process on the CO_2 content in the culture medium. As Kretschmer and Jacob (" Carbon dioxide requirement for outgrowth of Thermoactinomyces vulgaris spores" in Modarski, M., W. Kurylowicz, and J. Jeljaszewicz (Ed.) "Nocardia and Streptomyces", Gustav Fischer Verlag, Stuttgart - New York, 1978) found, growth of the germ tubes only occurs in the

presence of CO_2, which, however, can only accumulate sporadically in the required concentration due to the required aeration of the cultures. Even if the additives to the culture medium mentioned in WP 117 083 are used, with 6 TU/mL of culture medium, the enzyme yield is still too low from an economical standpoint with regard to production and application. The activity specification is in tyrosine units (TU), TU corresponding, according to TGL 29 166/02 1, to the amount of enzyme that liberates from a 0.625 % casein solution at pH 8.0 and 55 °C as many casein fragments soluble in 4 % trichloroacetic acid solution per 1 min as correspond to the absorption (274 nm) of 1 µmol of tyrosine.

OBJECT OF THE INVENTION

It is the object of the invention to avoid the mentioned disadvantages of the known methods by a reproducible germination and rapid and substantial growth of the organism in order to simultaneously effect an increase of the protease yield, and to guarantee a fermentation process that is easily controllable on a large scale, so that the economics of the process are improved under industrial conditions.

EXPLANATION OF THE ESSENCE OF THE INVENTION

The invention is based on the object to develop a method for producing thermitase, which guarantees reproducible growth of the microorganism with increased biomass yields and high enzyme synthesis efficiency.

In the method according to the invention for producing thermitase by culturing, pre-cultivation, and cultivation of microorganisms of the strain Thermoactinomyces vulgaris and/or the mutant thereof - deposited at the Central Strain Collection of the GDR in the Central Institute for

Microbiology and Experimental Therapy in Jena under IMET 9512 and IMET 9515 - one proceeds by inoculating an employed nutrient solution, which is saturated with oxygen, with spores for pre-cultivation and conducting the germination in a hermetically sealed vessel with a decrease of the oxygen partial pressure to 20 to 40 % of the oxygen partial pressure at saturation, preferably to 30 %, maintaining the desired oxygen partial pressure until germination of the spores is complete by introducing air, subsequently transferring the pre-culture into a fermenter with the production medium and carrying out the fermentation, additional quantities of nutrients being supplied during fermentation.

As production medium, corn starch, corn steep liquor, dry yeast, skimmed milk powder, sodium chloride, and calcium chloride are preferably used. It is advantageous to use starch hydrolysate and/or corn steep liquor hydrolysate. For pre-cultivation, i.e. the complete germination, it is very effective to maintain the oxygen partial pressure reached after the decrease in the pre-culture vessel for 2 to 4 h, preferably 2 h. For the actual production, it proved to be very convenient to supply corn starch or starch hydrolysates and/or corn steep liquor or corn steep liquor hydrolysates during fermentation in a quantity that corresponds to double or 1.5 times the quantity of the initial nutrient medium. The nutrients may be supplied continuously or in stages, beginning 2 to 6 h, preferably 2 h, after transferring the pre-culture into the fermenter. The invention is based on the knowledge that germination of the spores used as inoculum occurs reproducibly and to 100 % in a hermetically sealed culture vessel under elimination of gassing influences.

In detail, one advantageously proceeds by filling an agitated vessel, depending upon the required quantity of inoculum, preferably a glass flask with magnetic stirrer or

an agitated fermenter, with nutrient solution and sterilizing it. The vessel is equipped with a probe for measuring the oxygen partial pressure and with a regulating device for the addition of air. Prior to inoculation with spores, preferably with 10^7 - 10^8 spores/mL of nutrient solution, the nutrient solution is saturated with oxygen by introducing air. After inoculation, the vessel is hermetically sealed. The inoculated spores immediately begin to respire and thereby produce CO_2, which accumulates in the nutrient solution due to the lack of gas exchange with the environment. Within 30 - 60 min, the germ tubes grow out and the logarithmic development phase immediately follows. To avoid O_2 limitation and to guarantee adaptation of the pre-culture to the subsequent aerated fermentation stage, it is necessary to maintain the oxygen partial pressure after reaching 20 to 40 % of the partial pressure at saturation (initial value), preferably 30 %, at this level up to a period of 2 h to 4 h. After approximately 2 h to 4 h, the spores are 100 % germinated and already exhibit long, partially branched germ tubes, so that the pre-culture may be transferred into the production medium.

It has become apparent that this method of conducting the pre-culturing is essential for high reproducibility, and that in the fermenter, a growth and enzyme formation process proceeds uniformly and extremely effectively.

Measurement of the oxygen partial pressure during germination allows conclusions about the progress of this process and is consequently conducive to a reliable control (Fig. 1 shows the germination rate as a function of the oxygen partial pressure). The nutrient solution for performing the germination contains corn starch or starch hydrolysates, corn steep liquor or hydrolysates prepared from it, NaCl and $CaCl_2$. A composition of starch hydrolysate, corn steep liquor hydrolysate, NaCl, and $CaCl_2$ is advantageous, since it does not contain any insoluble

ingredients and hence allows exact microscopic control of the progress of the germination.

The enzyme yield in the production of thermitase is critically determined by the growth process, i.e. by the quantity of biomass formed and by the metabolic-physiological conditions at the stage of enzyme synthesis. In the method according to WP 117 083, an enzyme activity of 6 TU per mL of culture medium is not exceeded as a result of the limitation of C and N sources, since after 3 to 6 h of fermentation duration, the concentration of these substances required for growth and enzyme formation is no longer available. In particular the protein source or individual components thereof (amino acids) become limited. Performed tests have shown that by increasing the nutrient concentration at the beginning of the fermentation, an improvement is not achievable, since in this case the growth is inhibited for 3 to 4 h and for this reason the risk of infection increases again.

According to the invention, however, removal of this nutrient limitation with simultaneous avoidance of repressions of growth is possible if the required nutrients are supplied during fermentation. For this, it is necessary to supplement the C source to the specified extent and, in an adjusted ratio thereto, also the protein component. The level of C serves to maintain the energy balance and cell structure, while a defined concentration of N promotes the enzyme synthesis and maintains it longer.

It was found that the addition of the nutrients may occur in a continuous manner in the logarithmic growth phase, preferably starting from the 2^{nd} fermentation hour. A pulsed addition at intervals of 2 h, preferably starting from the 2nd fermentation hour, however, produces an effect that is equally good.

For the basic composition of nutrient solution and production medium, corn starch or starch hydrolysates, corn steep liquor or hydrolysates made thereof, yeast extract or dry yeast, NaCl, and $CaCl_2$ have proven to be beneficial. Addition of phosphates to increase the buffering action during sterilization is advantageous. Rapeseed oil and acetic acid are not generally required. As defoaming agent, commercially available silicone oils or vegetable oils (for example also rapeseed oil) may be used (0.1 %). As additional nutrients for the production medium, primarily corn starch, starch hydrolysates, and corn steep liquor are used. As already explained, by the end of the fermentation, the quantity of the additionally administered nutrients has to reach double to 1.5 times the initial concentration. It becomes apparent that with this procedure, the biomass concentration significantly increases compared to the method of WP 117 083. The enzyme synthesis is considerably increased, and for example with a fermentation period of 16 to 24 h achieves values of 10 to 12 TU/mL of nutrient solution compared to 6 TU/mL corresponding to the prior art.

A limitation of the nutrients manifests itself, among other things, in the breathing of the culture. Under constant aeration conditions, after reaching limiting substrate concentration, the oxygen partial pressure increases in the fermenter, i.e. breathing slows down. Additions of mixtures of carbohydrates and proteins then cause an immediate renewed increase of the O_2 consumption due to further growth and increased enzyme synthesis. In addition, the vigorous growth promotes the stability of the method with respect to detrimental outside effects, for example infections.

The method according to the invention is explained in more detail by means of the following examples.

EXAMPLARY EMBODIMENTS

Example 1

A 1 L round bottomed flask equipped with fittings for the incorporation of an O_2 sensor, for supplying sterile air, for withdrawing the air, and for inoculation, is filled with 800 mL of nutrient solution of the following composition and sterilized:

Starch hydrolysis product (SHP)	1.00% by mass
Corn steep liquor hydrolysate (approx. 7 % total protein content)	5.00% by mass
NaCl	0.10% by mass
$CaCl_2$	0.05% by mass

The content of the flask is mixed with a magnetic stirrer, maintained at a temperature of 50 °C, and saturated with oxygen by introducing air. From a homogenized spore suspension of the mutant IMET 9515, which was obtained by incubation for 20 hours of slant cultures on a corn steep liquor/starch medium at 56 °C and after elutriation with a 0.01 % Tween 80 solution, 10^7 spores/mL of nutrient solution, i.e. $8x10^9$ spores, are inoculated into the flask. Subsequently, the flask is hermetically sealed. Immediately after inoculation, breathing of the spores begins, which is detectably by the reduction of the O_2 concentration (Fig. 1). After approx. 85 min, the O_2 concentration (curve 1) in the flask has decreased to 30 % and air is supplied via a controlling device, so that this value can be maintained up to a time period of 120 min. The growing of germ tubes from the spores begins approx. 40 min after inoculation; after approx. 100 min, 100 % of the spores are germinated (curve 2). By monitoring the optical density, the beginning growth may be detected (curve 3). After 120 min, the culture is inoculated into a fermenter and the growth continues here with virtually no delay.

Example 2

A 30 L fermenter is filled with 20 L of production medium of the composition

Starch hydrolysis product (SHP)	1.0% by mass
Corn steep liquor hydrolysate (approx. 7 % total protein content)	5.0% by mass
NaCl	0.1% by mass
$CaCl_2$	0.05% by mass

and sterilized. Inoculation occurs with a pre-culture according to Example 1. The fermentation temperature is 50 °C, the stirrer has a rotational speed of 300 revolutions per minute, 300 L of air/h are supplied. As defoaming agent, 10 mL of silicone oil or rapeseed oil are added prior to inoculation. 2 h after the beginning of fermentation, the continuous supply of nutrients takes place, the nutrients consisting of

Starch hydrolysis product (SHP)	1.0% by mass
Corn steep liquor hydrolysate (approx. 7 % total protein content)	5.0% by mass

and that in a quantity of 2.5 mL/min. The metering is continued until the fermentation is finished after 26 h. Fig. 2 shows the result in comparison with fermentation without nutrient supply (basic medium of the above composition). The maximum biomass concentration increases in comparison with the control test (curve 1) from 1.7 mg/mL to approx. 3.5 mg/mL (curve 2). The enzyme synthesis proceeds with considerably increased formation rate and achieves in comparison with the control with 4 TU/mL of nutrient solution (curve 3) a value of approx. 10 TU/mL (curve 4).

Example 3

A 30 L fermenter is prepared with 20 L of production medium according to Example 2 and inoculated with a pre-culture according to Example 1. The fermentation parameters correspond to those from Example 2. After 2, 4, 6, 10, and 15 h, each time 200 mL of corn steep liquor hydrolysate are supplied. Fig. 3 shows the result. While the enzyme formation in the control is 4 TU/mL of medium, it increases to approx. 10.5 TU/mL when protein components are supplied (curve 2). The recorded values for the oxygen partial pressure in the fermenter (curve 3) clearly show the acceleration of the breathing after the 3rd, 4th, and 6th nutrient addition.

Example 4

A 30 L fermenter is prepared with 20 L of production medium of the composition

Corn starch	1.0% by mass
Corn steep liquor (50 %)	1.0% by mass
Dry yeast	0.03% by mass
Skimmed milk powder	0.05% by mass
NaCl	0.5% by mass
$CaCl_2$	0.05% by mass

according to Example 2 and inoculated with a pre-culture according to Example 1. The rotational speed of the stirrer is 400 revolutions per $minute^{-1}$, air is supplied with 300 L/h, the fermentation temperature is 50 °C. 2 h after the beginning of fermentation, continuous supply of additional nutrients takes place, the nutrients consisting of

Starch hydrolysis product (SHP)	1.0% by mass
Corn steep liquor hydrolysate (approx. 7 %	

total protein content) 5.0% by mass

in a quantity of 2.5 mL/min. The metering is continued until the fermentation is finished (24 h after the start of the test). Fig. 4 shows the enzyme formation (activity) as a function of time. After 24 h, it reaches 10.8 TU/mL of production medium.

EXHIBIT D

BP.PURA.017A-US.WO

PATENT CLAIMS

1. A method for producing thermitase by culturing, pre-cultivation, and cultivation of microorganisms of the strain Thermoactinomyces vulgaris and/or the mutant thereof - deposited at the Central Strain Collection of the GDR in the Central Institute for Microbiology and Experimental Therapy in Jena under IMET 9512 and IMET 9515 - characterized in that an employed nutrient solution, which is saturated with oxygen, is inoculated with spores for pre-cultivation and the germination is conducted in a hermetically sealed vessel with a decrease of the oxygen partial pressure to 20 to 40 % of the oxygen partial pressure at saturation, preferably to 30 %, the desired oxygen partial pressure is maintained until germination of the spores is complete by introducing air, the pre-culture is subsequently transferred into a fermenter with the production medium and the fermentation is carried out, additional quantities of nutrients being supplied during fermentation.

2. A method according to claim 1, characterized in that corn starch, corn steep liquor, dry yeast, skimmed milk powder, sodium chloride, and calcium chloride are used in the production medium.

3. A method according to claim 1 and 2, characterized in that starch hydrolysate and corn steep liquor hydrolysate are used.

4. A method according to claim 1, characterized in that the desired oxygen partial pressure in the pre-culture vessel is maintained for 2 to 4 h, preferably 2 h.

5. A method according to claim 1 and 2, characterized in that during fermentation, corn starch or starch

hydrolysates and/or corn steep liquor or corn steep liquor hydrolysates are supplied in a quantity that corresponds to double to 1.5 times the quantity of the initial nutrient medium.

6. A method according to claim 1 to 3, characterized in that the nutrients are supplied continuously or in stages, starting 2 to 6 h, preferably 2 h, after transferring the pre-culture into the fermenter.

Annexed...4... pages of drawings